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Marek's Disease Virus-Induced Skin Leukosis in Scaleless Chickens: Tumor Development in the Absence of Feather Follicles

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SUMMARY. Marek's disease virus (MDV) is an oncogenic cell-associated herpesvirus that causes T-cell lymphoma in chickens. Lymphoproliferative neoplasms in Marek's disease (MD) occur in various organs and tissues, including the viscera, peripheral nerves, skin, gonads, and musculatures. MDV is restrictively produced in the feather follicle epithelial (FFE) cells, and it gains access to the external environment via infected cells or as infectious enveloped cell-free virus particles. The goals of the present study were to 1) determine whether the MDV-induced skin lesions are neoplastic in nature or inflammatory reactions to viral infection, 2) determine whether physical presence of feather follicles (FF) is necessary for skin tumor development, and 3) study the role of skin epithelial cells not associated with feathers or FF in the replication and dissemination of infectious virus particles. Scaleless chickens that produce only a few scattered feathers and no sculate scales along the anterior metatarsi were used as a unique model to study the pathogenesis of dermal lesions. Histologic and immunohistochemical analysis revealed that the cutaneous lesions were tumorous as was manifested by massive accumulation of lymphoblasts and extensive activation of meq oncoprotein, the hallmark of MDV oncogenesis, within the skin lesions. Neoplastic cutaneous lesions in the scaleless chickens indicate that feather follicles are not necessary for skin tumor development. Finally, our preliminary data indicate that inoculation with supernatant fluid from homogenized and sonicated skin samples of MDV-infected scaleless chickens induces MD in susceptible birds, suggesting that skin epithelial cells not associated with FF also harbor infectious viral particles.

RESUMEN. Leucosis de la piel inducida por el virus de la enfermedad de Marek en aves sin escamas: Desarrollo de tumores en ausencia de folículos de la pluma.

El virus de la enfermedad de Marek es un herpesvirus oncogénico asociado a células que causa linfoma de células T en aves. Los neoplasmas linfoproliferativos en la enfermedad de Marek ocurren en varios órganos y tejidos, incluyendo las vísceras, los nervios periféricos, la piel, las gónadas y la musculatura. La producción del virus de la enfermedad de Marek se restringe a las células epiteliales del folículo de la pluma y el virus se disemina al medio ambiente por medio de las células infectadas o por medio de las partículas virales libres de células, con cobertura externa. Los objetivos del presente estudio fueron 1) determinar si las lesiones inducidas en la piel por el virus de la enfermedad de Marek son de naturaleza neoplásica o reacciones inflamatorias a la infección viral, 2) determinar si la presencia física de los folículos de la pluma es necesaria para el desarrollo de tumores en la piel y 3) estudiar el papel de las células epiteliales de piel no asociadas con el folículo de la pluma en la replicación y diseminación de las partículas infecciosas. Como un modelo único para estudiar la patogenesis de lesiones de la piel, se utilizaron aves sin escamas que producen solo escasas plumas esparcidas y no producen escamas en el metatarso anterior. Los análisis inmunológicos e inmunohistoquímicos revelaron que las lesiones cutáneas correspondían a tumores, como se evidenció por la acumulación masiva de linfoblastos y la extensa activación de la oncoproteína meq, que es característica de la oncogénesis generada por el virus de la enfermedad de Marek en las lesiones de piel. Las lesiones neoplásicas en la piel de aves sin escamas indican que los folículos de las plumas no son necesarios para el desarrollo de tumores de piel. Finalmente, nuestros datos preliminares indican que la inoculación con fluido sobrenadante homogenizado y sonicado, obtenido de muestras de piel de aves sin escamas infectadas con virus de la enfermedad de Marek, inducen la enfermedad de Marek en aves susceptibles, sugiriendo que las células epiteliales no asociadas al folículo de la pluma, también contienen partículas virales infecciosas.

Key words: Marek's disease, skin leukosis, scaleless chicken

Abbreviations: ADOL = Avian Disease and Oncology Laboratory; BTA = bursal-thymic atrophy; dpi = days postinfection; FF = feather follicles; FFE = feather follicle epithelium; PFU = plaque-forming unit; i.p. = intraperitoneal; MD = Marek's disease; MDV = Marek's disease virus; TP = transient paralysis

Marek's disease virus (MDV), a highly pathogenic and oncogenic avian α-herpesvirus, is the etiological agent of Marek's disease (MD), a contagious lymphoprolifereative disease of domestic chickens (4). MD is characterized by neoplastic lesions of the central and peripheral nerves, gonads, skin, kidneys, spleen, and liver (2,12). MDV spreads horizontally by gaining access to the external environment via infected feather follicle epithelial (FFE) cells or as infectious enveloped cell-free virus particles (3,24). The affected cells of the skin are the stratified squamous epithelium, which commonly slough off or detach with molted feathers, disseminating the virus in the environment (3).

MDV-induced dermal lesions that are generally obscured by feathers until inspection at the processing plants are one of the main reasons for condemnation and a major source of economical loss to the broiler industry. Since the initial observation by Helmbolt *et al.* (10), cutaneous lesions associated with MD have been described by many workers (5,6,7,11,17,22). Despite the earlier observation that MDV-induced skin tumors are lymphoid in nature, histologic and immunohistochemical details of these lesions are not adequately studied, and it remains to be determined whether skin epithelial cells undergo neoplastic alterations.

The activation of a limited number of viral genes in MD skin lesions has been demonstrated. MDV unique phosphoprotein 38 that is thought to play a role in the early cytolytic infection of

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Table 1. Skin sample collection for histological and immunohistochemical analysis.

dpi[A]	No. of infected birds tested	No. of control birds tested	No. of skin blocks tested/bird	Lesions	Skin sample used[B]	Meq activation[C]
7	2	2	3	−	Crural, abdominal, and sternal tracts	−
14	2	2	3	−	Crural, abdominal, and sternal tracts	−
23	2	2	3	+	Crural, abdominal, and sternal tracts	+
29	2	2	3	+	Dorsal cervical and capital tracts	+
36	2	2	3	+	Upper and under prepatagial apterium	+
41	2	2	3	−	Dorsal cervical and capital tracts	−

[A]Skin samples from the infected and control scaleless chickens were collected at 7, 14, 23, 29, 36, and 41 dpi for histologic and immunohistochemical analysis.

[B]Skin sections used for histology and antigen activation studies.

[C]The expression level of meq oncoprotein was based on the age and severity of tumor nodules. Based on an arbitrary system, the expression levels were scored from 1+ (lowest) to 4+ (highest). Samples tested at 14 dpi were mainly 1+. Samples tested at 23 dpi were 2−3+. Samples tested at 29 dpi were all 4+. The level of expression at 36 dpi was 1−2+. Negative sign is an indication of no expression.

lymphocytes and maintenance of transformation (25) was shown to be highly expressed in the experimentally induced MD cutaneous lymphoma lesions (5). The activation of meq, a major latency/transformation antigen, has been studied in the viscera and nerves but not the skin of MDV-infected chickens (8,9).

In the present study, the development of skin lesions, physical association between cutaneous nodules and feather follicles (FF), and activation pattern of meq, a homolog of *fos* and *jun* oncogenes required for tumor development and maintenance (13,15,16), were investigated in the skin samples of MDV-infected scaleless chickens. These scaleless chickens carry a recessive autosomal mutation in the scale gene (sc) that in the homozygous state (sc/sc) produce only a few scattered feathers over the body and no sculate scales along the anterior metatarsi (1,18,20). To determine whether the infectious virus particles are replicated within the epithelial cells of the "featherless" skin of the scaleless chickens, the supernatant and the filtrate of the homogenized and sonicated skin samples of MDV-infected scaleless chickens devoid of any visible feathers or FF were inoculated into MD-susceptible chickens, and the birds were observed for 8 wk for clinical signs and tumor development.

MATERIALS AND METHODS

Experimental birds. The scaleless chicken embryos were purchased from the Department of Poultry Genetics, University of Connecticut, Storrs, CT. These birds carry vaccination-induced maternal antibodies against MDV and turkey herpesvirus. The feathered chickens were F_1 progeny (15X7) of Avian Disease and Oncology Laboratory (ADOL, East Lansing, MI) Line $15I_5$ males and 7_1 females. The 15X7 birds carried no maternal antibody against MDV, and they were susceptible to MD. Chicks were hatched at ADOL poultry facility and housed in modified Horsfall-Bauer isolation units for the duration of the experiment. All birds were wing-banded at hatch and sorted randomly into four groups. Chickens were observed for clinical signs and development of cutaneous lesions.

Virus. A very virulent plus (vv+) strain of MDV, 686, that is propagated and maintained in our laboratory was used in this experiment (23).

Histopathology. Skin samples were collected and immersed in 10% neutral buffered formalized saline solution. After dehydration in graded ethanol solutions, samples were embedded in paraffin, sectioned, and mounted on glass slides for hematoxylin and eosin staining.

Immunohistochemistry. An avidin-biotin-peroxidase complex (ABC kit, Vectastain; Vector Laboratories, Burlingame, CA) was used for immunohistochemical studies. Microtome sections were mounted on poly-L-lysine-coated slides and fixed in acetone for 45 min. After 15 min of equilibration in phosphate-buffered saline, samples were pretreated for 20 min with normal horse serum (provided with the kit) to decrease nonspecific antibody binding. Samples were then incubated with the primary antibody for 30 min at room temperature followed by rinsing and incubation with the secondary antibody for 30 min (provided in the kit). The MDV antigen localization was visualized by incubation of the sections with 3,3-diaminobenzidine-H_2O_2 solution. All sections were then counterstained with hematoxylin followed by alcohol washes and Pro-par clearant (Anatech Ltd., Battle Creek, MI). The working dilution for the polyclonal antibody specific for meq was 1:2000. The antibody to meq oncoprotein was developed and characterized at the ADOL (14).

Preparation and inoculation of skin sample. Skin sample from the dorsal cervical and capital tracts of a scaleless bird devoid of any visible feather or FF with extensive lesion at 29 days postinoculation was collected into 4 ml of culture medium at 4 C. Sample was homogenized in a hand-held glass homogenizer and centrifuged at 2000 × *g* for 20 min. The supernatant was harvested, and the pellet was resuspended in 4 ml of the same medium at 4 C. The pellet suspension was sonicated for three 20-sec cycles, with 60-sec pause between cycles, by using the 40T probe of a Braun-Sonic 2000U sonifier (B. Braun Biotech Inc., Allentown, PA) at a power setting of 24. The sonicated sample was centrifuged as described above, and the supernatant was collected. Then, 1.5 ml of the supernatant from the sonicated sample was passed through a 0.45-μm filter, and the filtrate was collected for bird inoculation. One hundred microliters from each preparation was used for intraperitoneal (i.p.) inoculation of MD susceptible birds.

Experimental design. One-day-old scaleless chicks were randomly distributed into 4 groups of 15 birds each in separate isolators (A1, A2, B1, and B2). Birds in groups A1 and A2 were inoculated subcutaneously with 2000 plaque-forming units (PFU) of 686-MDV at 1 wk of age. Birds in groups B1 and B2 served as negative noninoculated controls. Selected birds from the infected scaleless chickens (group A1 or A2) were euthanized by CO_2 inhalation and necropsied along with uninfected age-matched controls (group B1 or B2) at different days postinfection (dpi), and skin samples with and without lesions were taken for histologic and immunohistochemical analysis (Table 1). The selection process was based on clinical signs and presence or absence of skin lesions. For skin sample inoculation, 40 chickens from Line $15I_5X7_1$ with no maternal anti-MDV antibody were randomly distributed into four groups of 10 birds each in separate isolators (A, B, C, and D). Seven birds in group A received 100 μl each of the supernatant from the homogenized preparation by i.p. inoculation. Seven birds in groups B and C received 100 μl each of the supernatant from the sonicated and filtrated samples, respectively. The remaining three birds in each group served as contact birds. The chickens in group D received medium only and served as noninfected control birds. All birds were inoculated at 1 wk posthatch.

RESULTS

The scaleless chickens were inoculated subcutaneously with 2000 PFU of 686-MDV 1 wk after hatch, and they were observed for

Fig. 1. Skin leukosis induced by MDV. Chicks were inoculated subcutaneously with 2000 PFU of 686-MDV 1 wk posthatch. Skin lesions developed approximately 3 wk postinoculation. This picture depicts lesions developed in the dorsal cervical and capital tracts of a 5-wk-old chick.

Fig. 2. Skin leukosis induced by 686-MDV in the femoral, sternal, and abdominal tracts of a 6-wk-old scaleless bird.

clinical symptoms and skin lesions development. The scaleless chickens were susceptible to MD, and they developed typical clinical signs associated with MD. Skin lesions developed approximately 3 wk postinoculation. The 15X7 birds inoculated with different skin sample preparations from the infected scaleless bird, developed transient paralysis (TP) around 8 dpi followed by depression, crippling, and weight loss.

Gross lesions. Skin lesions developed in approximately 40% of the inoculated birds with initial appearance at about 3 wk postinoculation. Lesions exhibited varying degrees of nodular enlargement with initial glistening appearance. No regressive type of skin lesions that would indicate an inflammatory reaction rather than tumor development was observed in the infected scaleless chickens. Table 1 shows the time schedule for skin sample collection from the infected and control scaleless chickens during the cytolytic, latency, and reactivation phases of MDV infection. Fig. 1 depicts MDV-induced cutaneous lesions developed in the dorsal cervical and capital tracts of a 5-wk-old scaleless chick. Fig. 2 shows skin leukosis induced by 686-MDV in the femoral, sternal, and abdominal tracts of a 6-wk-old scaleless bird. Cutaneous lesions induced by MDV can be observed in different parts of the body as early as 3 wk postinoculation, and afflicted birds generally exhibit gross involvement of visceral organs.

Histopathology. Fig. 3 shows the histologic analysis of the cutaneous lesions with condensed cell populations in the abdominal tract of a 6-wk-old infected featherless bird (indicated by arrows). Most of the lesions are developed in the absence of any visible feather or FF adjacent to the tumor nodules. Fig. 4A shows higher magnification (400×) of the skin lesions in Fig. 3 with massive accumulation of neoplastic and lymphoid cell populations. Fig. 4B is the histologic section of the skin sample of a mock-infected control bird. This slide depicts the normal distribution of cell population in the skin of an uninfected scaleless bird.

Immunohistochemistry. Meq is a major latency/transformation oncogene that is expressed in all MDV-induced lymphoblast tumor cells (13,15). Fig. 5A shows the extensive expression of meq within the MDV-induced skin lymphomas of a male bird at 29 dpi. Meq is necessary for tumor development, and it is a key gene associated with MDV pathogenecity (16). Fig. 5B shows the immunohistochemical staining of meq antigen in a noninfected scaleless chicken.

Skin sample inoculation. Table 2 shows the results from i.p. inoculation of MD-susceptible birds with different preparations of skin sample from an infected scaleless bird. Five of seven birds in group A receiving supernatant of homogenized sample died from MD-induced (TP) or other complication before termination at 8 wk postinoculation. All the dead birds had moderate-to-severe bursal and thymic atrophy (BTA). The two surviving birds looked healthy, and they were negative for MD at termination. Only one of the contact birds in this group died from TP with BTA. The other two birds survived for the duration of the experiment, and they showed no complication associated with MD. All the infected and contact birds in group B died from MD complication before termination. Two of the infected birds had MD, and the remaining infected and contact birds had moderate-to-severe BTA. All the inoculated and contact birds in group C died before termination at 8 weeks postinoculation. Necropsy revealed that two of the inoculated birds had developed MD and that five birds had BTA. All three contact birds died with BTA and one bird with enlarged vagus nerve. All the

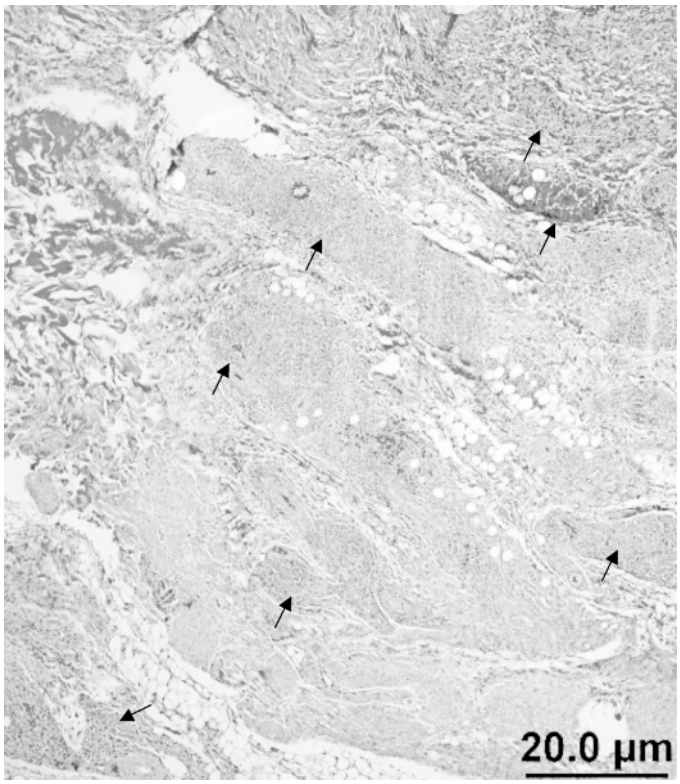

Fig. 3. Histologic analysis of skin leukosis associated with MD. This slide depicts the extensive cutaneous lesions induced by MDV in the skin on the abdomen of a 6-wk-old female scaleless bird (indicated by arrows).

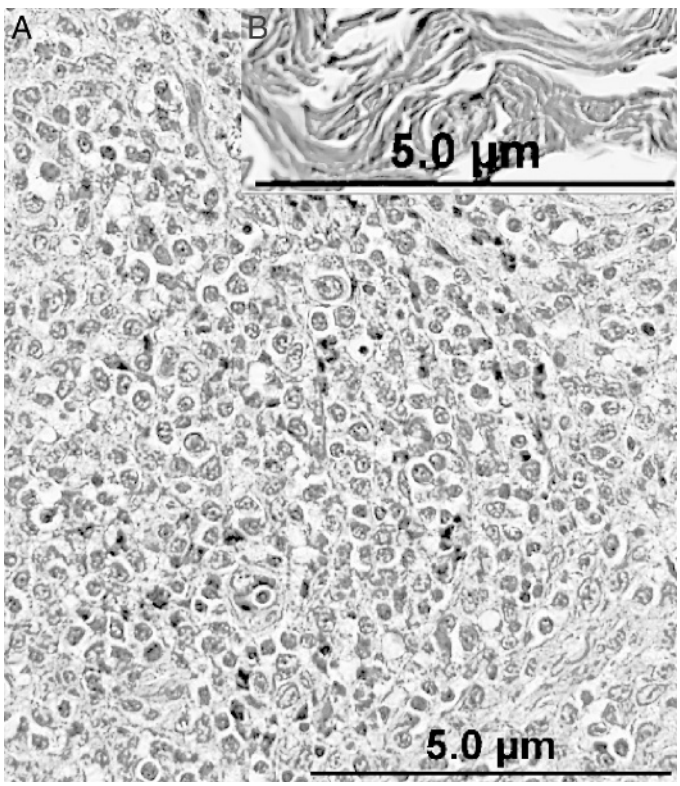

Fig. 4. (A) High-power magnification (400×) of skin sample in Fig. 3 showing massive infiltration consisting mainly of lymphoblasts with few heterophils and occasional MD cells. (B) Normal distribution of cell population in the skin sample of a 6-wk-old uninfected scaleless bird.

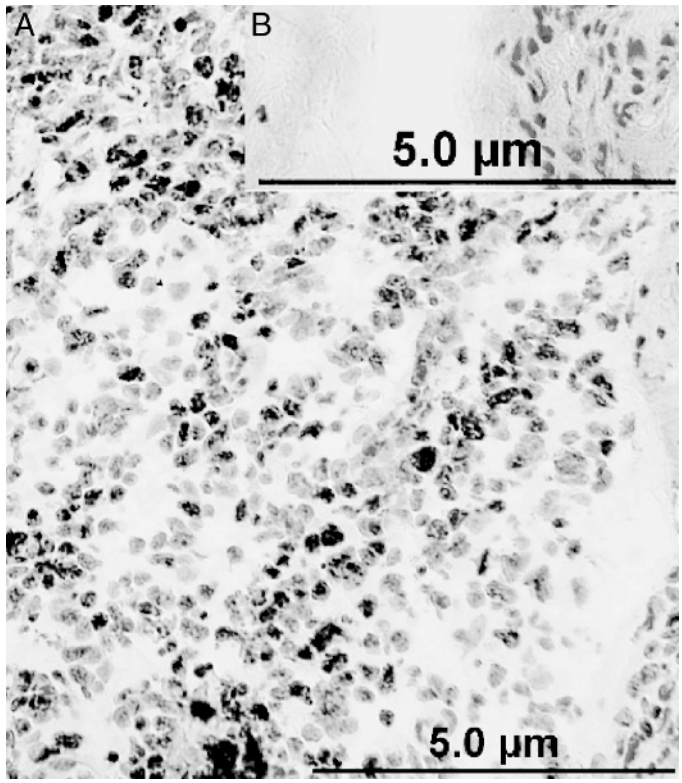

Fig. 5. Meq expression in skin leukosis. (A) The meq oncogene associated with latency/transformation is heavily expressed in the transformed lymphoblasts of the skin lesions of a male bird at 29 dpi. (B) The absence of meq antigen in the pectoral tract of an uninfected 36-day-old bird.

mock-infected control birds in group D survived for the duration of the experiment without any clinical symptoms.

DISCUSSION

Infectious enveloped MDV is restrictively produced in the FFE cells of infected chickens, and it is disseminated into the environment via dander and feather dust (3,24). The scaleless chickens used in this study carry a recessive autosomal mutation in the scale gene, which results in the production of only a few scattered feathers and no sculate scales in the homozygous state (1,18,20). Despite the lack of normal plumage and FF, the scaleless chickens are capable of horizontal transmission of MDV to contact cage-mates. Our previous studies showed that the rate of horizontal transmission of MDV was delayed by 4 days in the scaleless chickens in comparison with their feathered hatch-mates (data not shown). It is not clear whether the FFE cells encasing the shafts of the few existing feathers are responsible for the replication and spread of MDV, or whether epithelial cells in general are uniquely equipped for such an intriguing task. It has been shown that the affected stratified squamous epithelial cells of the skin in the infected chickens commonly slough off and spread the infectious virus particles into the environment (3). It is likely that FF only act as gateways to facilitate the dissemination of MDV into the environment, and FFE and epithelial cells of the skin not associated with feathers or FF are both capable of supporting replication and production of MDV.

Our preliminary data indicate that inoculation with supernatant from homogenized and sonicated skin samples of MDV-infected

Table 2. Early mortality and tumor development after inoculation of chickens with skin sample preparations.

Sample prep in culture medium	Treatment	Inoculated birds			Contact birds		
		Total no. of birds	MD death before termination	MD at termination	Total no. of birds	MD death before termination	MD at termination
Dorsal-cervical and capital tracts	Homogenized[A]	7	5	0	3	1	0
Dorsal-cervical and capital tracts	Homogenized and sonicated[B]	7	7	0	3	3	0
Dorsal-cervical and capital tracts	Filtered (0.45 μm)[C]	7	7	0	3	3	0
Medium only	None	7	0	0	3	0	0

[A]Dorsal cervical and capital tracts of a scaleless bird at 29 dpi with extensive lesions was homogenized and centrifuged at 2000 × *g* for 20 min. One hundred microliters of the supernatant was inoculated i.p. into each antibody-negative MD-susceptible bird ($15I_5X7_1$) 1 wk posthatch.

[B]The pellet from the homogenization step was further sonicated and centrifuged as before. One hundred microliters of the supernatant was used for i.p. inoculation of each bird.

[C]The supernatant (1.5 ml) from the sonicated sample was passed through a 0.45-μm filter, and 100 μl of the filtrate was used for i.p. inoculation of the birds as before.

scaleless chickens free of FF induces MD in susceptible birds. Also, filtrate of this supernatant passed through a 0.45-μm filter was shown to contain infectious virus particles (Table 2).

The histologic studies showed that the MDV-induced chronic progressive skin lesions were tumorous indeed as manifested by massive infiltration of lymphoid cells consisting mainly of lymphoblasts (Figs. 3, 4A) and extensive activation of meq oncoprotein (Fig. 5A). No regressive types of lesions reported previously (6) were observed in this study. It was also apparent from the gross lesions in the skin of the scaleless chickens that tumor development is not necessarily associated with the presence of FF. Figs. 1 and 2 clearly show the induction of extensive lesions in the absence of feathers and FF in the vicinity of tumor nodules. Microscopic analysis of featherless skin samples from the scaleless chickens with or without lesions revealed no rudimentary FF that could be involved in the replication and dissemination of MDV (Fig. 3). The role of the nonmigratory FFE cells is probably limited only to the replication, production, and spread of the infectious virus particles into the environment. The dissemination of the virus within the skin tissue, however, is a key role likely played by the MDV-infected $CD4^+$ $CD8^-$ cells migrating and delivering infectious virus particles from the venous circulation and inducing skin tumors (7,21).

Meq, a MDV-specific oncogene with a basic leucine zipper structure, is consistently expressed in all tumor samples and MD lymphoblastic cell lines examined (19). Recently, it has been shown that meq is a homolog of *fos* and *jun* oncogenes with antiapoptotic property and that it is required for tumor development and maintenance (13,15,16). Furthermore, deletion of a functional meq gene was found to be indispensable for MDV oncogenicity but not virus replication (16). The immunohistochemical analysis in this study revealed the extensive expression of meq oncoprotein within all the tested tumor samples. Fig. 5A depicts the massive accumulation of lymphoblasts and the extent of meq antigen up-regulation within the transformed cells. Meq antigen expression was not detected during cytolytic and latency infections (Table 1). All the nontumorous skin samples were negative for meq oncoprotein activation.

In summary, our data indicate that the chronic progressive type of skin lesions induced by MDV are neoplastic in nature as was demonstrated by extensive lymphoblast aggregates and high level expression of meq oncoprotein. In addition, it was shown that the physical presence of feathers or FF is not necessary for the cutaneous lesion development. Furthermore, our preliminary studies indicate that it is not only the FFE but also skin epithelial cells not associated with feathers that are capable of supporting the replication and possibly the dissemination of the virus particles.

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